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Specification and Drawings, as originally filed, with Application for Patent Serial No: **2,260,376,** on February 11, 1999, by **UNIVERSITE DE MONTRÉAL,** assignee of Luc Desgroseillers and Guy Boileau, for "New Metalloproteases of the Neprilysin Family".

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ABSTRACT

In this paper, we describe RT-PCR strategies that allowed us to identify and clone new members of the NEP-like family. Degenerate oligonucleotide primers corresponding to consensus sequences located on either side of the HEXXH consensus sequence for zincins were designed and used in RT-PCR with mouse and human testis cDNAs. DNA fragments with lengths expected from the sequence of this class of enzymes were obtained. These DNA fragments were cloned and sequenced. Using this PCR strategy and the PCR fragments as probes to screen cDNA libraries, three new zincin-like peptidases were identified in addition of known members of the family. The cDNA sequences allowed to derive specific probes for Northern and *in situ* hybridization, and probe human chromosomes to localize the gene and establish potential links to genetic diseases. Furthermore, these cDNA sequences were used to produce recombinant fusion proteins in *Escherichia coli* in order to raise specific antibodies. Finally, the cDNA sequences were cloned in mammalian expression vectors and transfected in various mammalian cell lines to produce active recombinant enzymes suitable for testing specific inhibitors.

TITLE OF THE INVENTION

New Metalloproteases of the Neprilysin Family

BACKGROUND OF THE INVENTION

Peptides are used by cells from yeast to mammals to elicit physiological responses. The use of peptides as messengers usually involves the following steps: 1) production and release of the peptide by a specific cell, 2) interaction of the peptide with a receptor on the surface of the target cell, and 3) degradation of the peptide to terminate its action. The first and last steps of this scheme require the participation of proteases/peptidases. There is increasing evidence that membrane-associated zinc-metallopeptidases play important roles in both of these steps. Although activation of prohormone precursors into bioactive peptides is generally performed by proteases of the subtilisin family located in the *Trans*-Golgi Network or in secretory granules of the cell (for a review see: (Seidah and Chrétien, 1995)) a few peptides need a final processing step. This step involves the action of membrane-associated zinc-metallopeptidases. Two cases are particularly well documented: angiotensin-converting enzyme (ACE) which cleaves inactive angiotensin I into angiotensin II (Corvol and Williams, 1997) and endothelin-converting enzymes (ECEs) which cleave isoforms of big endothelins into endothelins (Turner, 1997a). In addition to their role in peptide activation, cell surface zinc-metallopeptidases have also been implicated in the termination of the peptidergic signal by breaking down the active peptides into inactive fragments. One of the best known of these peptidases is probably Neutral Endopeptidase-24.11 (Neprilysin, NEP) that has been implicated in the physiological degradation of several bioactive peptides (Kenny, 1993). Interestingly, NEP and the ECEs show significant structural similarities and appear to be members of a family of peptidases that also includes PEX, a newly discovered and not yet characterized peptidase, and the KELL blood group protein (Turner and Tanzawa, 1997b). Because of their important role as regulators of bioactive peptide activity, these enzymes (more specifically NEP and the ECEs) have been identified as putative targets for therapeutic intervention, similar to the way ACE inhibitors are used to control blood pressure. The recent discovery of PEX, another member of the family, which appears to be involved in phosphate homeostasis, raised the possibility that other yet unknown members might exist.

Members of the NEP-like family are type II membrane proteins consisting of three distinct domains: a short NH2-terminal cytosolic sequence, a single transmembrane region, and a large extracellular or ectodomain responsible for the catalytic activity of the enzyme. There are potential N-glycosylation sites and cysteine residues that are involved in disulfide bridges stabilizing the conformation of the active enzyme. These enzymes are metalloenzymes with a Zn atom in their active site. As

such, they belong to the zincin family of peptidases which is characterized by the active site consensus sequence HEXXH (Hooper, 1994), where the two histidine residues are zinc ligands. In members of the NEP-like family of peptidases, the third zinc ligand is a glutamic acid residue located on the carboxy-terminus side of the consensus sequence. This characteristic puts them in the gluzincin sub-family (Hooper, 1994). The model enzyme for gluzincins is thermolysin (TLN) a bacterial protease whose 3D structure has been determined by X-ray crystallography (Holmes and Matthews, 1982). The active site of NEP has been extensively studied by site-directed mutagenesis and several residues involved in zinc binding (Devault et al., 1988b; Le Moual et al., 1991; Le Moual et al., 1994), catalysis (Devault et al., 1988a; Dion et al., 1993), or substrate binding (Vijayaraghavan et al., 1990; Beaumont et al., 1991; Dion et al., 1995; Marie-Claire et al., 1997) have been identified (for a recent review see (Crine et al., 1997).

SUMMARY OF THE INVENTION

Here, we developed an RT-PCR strategy to look for other members of this important family of peptidases. This strategy allowed the molecular cloning and characterization of three additional NEP-like (NL) metallopeptidases (called NL-1, NL-2 and NL-3). Knowledge obtained through these studies will allow the generation of reagents (nucleic acid probes, antibodies and active recombinant enzymes) for further biochemical characterization of these enzymes and their pattern of expression and will greatly help the rational design of specific inhibitors that could be used as therapeutic agents.

The present invention will be described hereinbelow by referring to specific embodiments and appended figures, which purpose is to illustrate the invention rather than to limit its scope.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Amino acid sequence comparison of human NEP, PEX, KELL and ECE1 peptidases. Amino acid sequences in boxes are those used to design the oligonucleotide primers. Numbers and arrows under the sequences identify the primer and its orientation.

Figure 2: Sequences of the oligonucleotide primers used in the PCR reactions.

Figure 3: Nucleotide and amino acid sequence of the mouse NL-1 cDNA. The sequence of the DNA fragment obtained by PCR is in brackets.

Figure 4: Partial nucleotide and amino acid sequence of the human NL-2 cDNA. The sequence of the DNA fragment obtained by PCR is in brackets.

Figure 5: Partial nucleotide and amino acid sequence of the human NL-3 cDNA.

Figure 6: Amino acid sequence comparison of NEP, NL-1, NL-2 and NL-3 peptidases.

Figure 7: *In situ* hybridization of mouse testis sections using NL-1 as a probe.

Figure 8: *In situ* hybridization of mouse sections using mouse NL-3 as a probe.

Figure 9: *In situ* hybridization of mouse spinal chord sections

Figure 10: Expression of NL-1 in mammalian cells.

Figure 11: Activity of recombinant soluble NL-1.

Figure 12: Expression of a soluble form of NL-3 using NL-1 amino-terminal domain.

DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION

MATERIALS AND METHODS

DNA and RNA manipulations

All DNA manipulations and Northern blot analysis were performed according to standard protocols (Ausubel et al., 1988; Sambrook et al., 1989).

mRNA purification and cDNA synthesis

mRNAs were prepared from mouse testis using Quick Prep Micro mRNA purification kit (Pharmacia Biotech). Purified mRNAs were kept at -70° until ready used. First strand cDNA was synthesized from 1µg of mRNA using the First-Strand cDNA synthesis kit (Pharmacia Biotech). The human testis cDNA library was obtained from Clonetech.

Polymerase chain reaction protocol

PCR was performed in a DNA thermal cycler with 5 µl of cDNA template and 1 µl of Taq DNA polymerase in a final volume of 100 µl, containing 1 mM $MgCl_2$, 2 µM of each primer oligonucleotide, 20 µM of each dNTP and 5% DMSO. Cycling profiles included an initial denaturation step of 5 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 40°C and 1.5 min at 72°C. A final extension step was performed at 72°C for 10 min. The amplified DNA was loaded on a 2% agarose gel and visualized by staining with ethidium bromide. Fragments ranging in size between 500-700 bp were cut and eluted from the gel. If needed, a second round of PCR was done with nested oligonucleotide primers, using 10 µl of the first PCR reaction, or of the eluted band cut from the agarose gel. Resulting fragments were ligated in pCR2.1 vector (Invitrogen) according to the distributor's recommendations. DH5α *Escherichia coli* cells were transformed with the ligation mixture and grown on 2YT plates in the presence of kanamycin. Plasmids were prepared from resistant cells and sequenced.

In situ hybridization on mouse tissues and chromosomal localization of human genes

In situ hybridization on whole mouse slices or isolated tissues was performed as described previously (Ruchon et al., 1998).

To determine the chromosomal localization of human NL-2 and NL-3 genes, a

technique for mapping genes directly to banded human chromosomes was used. Metaphase chromosomes were obtained from lymphocytes cultured from normal human peripheral blood. Cells were synchronized with thymidine and treated with 5-bromodeoxyuridine (BrdU) during the last part of the S phase to produce R-banding. Biotin-labeling of the probe was done by nick-translation (Bionick, BRL) and the probe was visualized by indirect immunofluorescence.

Antibody production

To raise antibodies against the new peptidases, the cDNA sequences of each protein was compared to that of other members of the family and the sequence segment showing the less homology was used. These sequences are from amino acid residues 273 to 354 for NL-1, from xx to xx for NL-2 and from 143 to 465 for NL-3. These cDNA fragments were cloned in vector pGEX2T (Pharmacia Biotechnology) downstream from and in phase with Gluthatione-S-transferase (GST). Plasmids were transformed in *E. coli* strain AP401 and, induction of synthesis and purification of the fusion proteins were performed as recommended by the supplier. The NL polypeptides were cleaved from the fusion protein with thrombin and purified by SDS-PAGE. NL polypeptides were injected to rabbits or mice according to the following schedules: for rabbits, initial injection of 150 µg of protein with boosts of the same amount 4 weeks and 8 weeks following the initial injection; for mice, initial injection of 100 µg of protein followed by boosts of the same amounts 3 and 6 weeks later. A month after the last injection, sera were collected from the animals and tested by immunoblotting against the initial *E. coli* produced antigens and the recombinant proteins produced in mammalian cell lines.

Expression of NLs in cultured mammalian cells and enzymatic assays

The cDNAs for NL-1 and NL-3 were cloned in vectors pcDNA3 or pRcCMV (Invitrogen) and introduced by transfection in mammalian cell lines according to procedures already described in our laboratory (Devault *et al.*, 1988a). Procedures to prepare extracts of cellular proteins or culture media were also described in previous papers (Devault *et al.*, 1988a; Lemay *et al.*, 1989). The presence of NLs in these extracts was monitored by immunoblotting using specific antibodies.

Extracts of cellular proteins and culture media were assayed for enzymatic activity. Two tests were performed. The first used [^{3}H]-Tyr-(D)Ala$_2$-Leu-enkephalin as substrates and was performed according to Lemay *et al.* (1989). The second used big endothelin-1 as substrate and was performed as described previously (xx). **RESULTS**

Cloning of NL-1, a new member of the NEP family

The molecular cloning in the past few years of ECEs, PEX and KELL showed that all these proteins have between 50 and 60% similarity with NEP. This observation led us to believe that these peptidases are part of an extended family and that there could be still additional members to be discovered. To test this hypothesis, we aligned

the amino acid sequences of the members of the NEP-like family and designed degenerate oligonucleotide primers to be used in RT-PCR reactions (Figure 1 and 2). These primers were located on either side of the HEXXH consensus sequence for zincins. Because they are highly degenerate, primers 1 and 2 were each subdivided into two pools, 1A-1B, and 2A-2B, respectively (Figure 2). Any PCR amplified DNA fragment that corresponds to a peptidase of the family should normally contain the consensus sequence and be easily recognized by sequencing of the cloned fragments. Using this strategy, we first performed PCR reactions with primer pairs 1A-3 and 1B-3. The amplified DNA migrates mostly as a smear starting at around 700 bp and going down to 100 bp. As the expected fragments should be around 550 bp, we isolated from the gel the section corresponding to DNA fragments longer than 500 bp. A second round of PCR reactions was performed with both crude PCR products of the first reaction and isolated DNA bands, using primers 2A-3 and 2B-3. The expected 296 bp fragment was seen on the gel (not shown).

Cloning of these DNA fragments generated approximately 350 clones, of which 44 were sequenced. Nine of these had no inserts or corresponded to sequences not related to the NEP family, 24 corresponded to NEP, 3 to PEX, and 8 corresponded to one putative new member of the family, since they all contained the HEXXH consensus sequence for zincins and showed 65% homology with mouse NEP (in boxes Figure 3). This fragment was then used to screen a mouse testis cDNA library, and allowed us to isolate a complete cDNA of 2592 nucleotides (Figure 3). The identity of this sequence with other members of the family is presented in Table I. This new member was called NL-1, for NEP-like peptidase 1. This sequence is not present in publicly accessible DNA data banks.

Cloning of NL-2 and NL-3.

A strategy similar to that described for amplification of enzymes of the NEP family from mouse testis cDNAs was used with a human testis cDNA library using two different oligonucleotide primers. This time, DNA fragments of approximately 900 bp were obtained and cloned. Ten clones were sequenced, revealing the presence of NEP and two new peptidases of the family that we have called NL-2 and NL-3.

The NL-2 PCR fragment was 879 nucleotides in length and encoded a 293 amino acid residue segment probably located in the carboxy-terminal domain of this putative peptidase (in brackets Figure 4). This PCR fragment was then used to screen a lambda gt10 human brain cDNA library. It allowed the isolation of a 1827 bp cDNA fragment which covers 609 amino acids of NL-2 ORF and overlaps partially with the NL-2 PCR fragment. Fusion of this lambda clone and the PCR fragment resulted in an open reading frame of 706 amino acid residues. The use of 5' RACE protocols with human testis cDNA libraries allowed completion of the sequence of NL-2 ORF (Figure 4). This ORF codes for a putative protein that is 77.4% identical to the mouse NL-1

protein (Figure 6). Across species, members of the NEP, PEX, ECEs sub-families have highly conserved sequences (more than 94% identity). Thus a sequence identity of 77.4% only, between the novel human protein and mouse NL-1 suggests that this protein is not the human homologue of NL-1, but appears to be another member of the family. The identity of NL-2 with other members of the family is presented in Table I. NL-2 sequence was not found in publicly accessible DNA data banks.

The 879 bp PCR fragment encoding NL-3 showed an open reading frame of 293 amino acid residues (Figure 5, in brackets). Sequence analysis of NL-3 showed that it was 94.2 % identical to an EST sequence from mouse embryonic tissue present in publicly accessible DNA data banks. This mouse EST sequence, commercially available from American Tissue and Cell Culture (ATCC), had been obtained previously by our laboratories.

Since Northern blot analysis of human tissues with the NL-3 PCR fragment showed the expression of this protein in spinal chord (see below), the same PCR DNA fragment was used to screen by hybridization a human spinal chord cDNA library constructed in phage λ vectors. One clone contained a full-length ORF of 752 amino acid residues that encompassed the 293 amino acid residue ORF of the PCR fragment. The sequence of NL-3 is currently being finished. Figure 5 shows NL-3 partial sequence.

Figure 6 presents a comparison of the amino acid sequence of the new NEP-like enzymes and Table I shows the extent of identity between members of the family.

Cellular distribution of NL-1, NL-2 and NL-3 peptidases

Determining the tissue distribution of NL-1, NL-2 and NL-3 may provide clues to identify the peptidergic systems in which they are involved. It will be particularly interesting to compare the tissue distribution of these peptidases with that of NEP and the ECEs to determine whether or not the physiological functions of NL-1 and/or NL-2 and/or NL-3 may overlap those of NEP and/or ECEs.

In situ hybridization (ISH), using our mouse cDNA, was used to determine the spatial and temporal expression of NL-1 during mouse development, as done previously for PEX (Ruchon et al., 1998)). Serial sections of whole foetal (12, 15 and 19 dpc) and adult mice (1, 3 and 6 days old) were hybridized with an [^{35}S]-labeled RNA probe. Figure 7 shows a section of mouse testis which was the only tissue identified to express NL-1 by this technique. Cells of seminiferous tubules are specifically labeled but spermatids located near the center of the tubule showed strongest labeling. These cells are in the last stage of maturation into spermatozoids. The presence of NL-1 in testis has now been confirmed by Northern analysis of mouse tissues (see Fig. 10).

A similar approach was used to determine the localization of NL-3 using the mouse EST obtained from ATCC. Figure 8 shows sections of whole mouse at 17 days of embryonic development and 4 days post-natal. Several tissues are expressing this

putative peptidase including brain, where it is associated with neurons (Figure 9), spinal chord, liver, spleen and bones. Labeling was stronger in bones from *Hyp* mouse, an animal model for hypophosphatemic rickets (Figure 8). In bones, NL-3 was found to be expressed by osteoblasts (not shown).

Northern blotting experiments were performed on several tissues with NL-2 and NL-3 probes. A Human Multiple Tissues Northern Blot (Clontech) was hybridized with specific probes. A single RNA band of approximately 4.0 kb was revealed by the probe for NL-2. Expression of NL-2 is restricted to brain and spinal cord (not shown). However, RT-PCR has shown the presence of this enzyme in testis (not shown).

A single RNA band of approximately 3.0 kb was detected with the specific probe for NL-3 (not shown). NL-3 expression was observed mainly in ovary, spinal cord and adrenal gland.

Chromosomal localisation of the human gene for NL-2 and NL-3

As a mean to get clues on the function of the new metallopeptidases in vertebrates, we have localized the new cDNAs on human chromosomes, in order to look for a possible link between the gene locus and mapped genetic diseases in humans. To do so, we have mapped the NL-2 and NL-3 genes by high-resolution fluorescence *in situ* hybridization (FISH). NL-2 was localized to chromosome band 1p36. Consistent with the cellular distribution of NL-2 in humans, genetic diseases of the CNS such as dyslexia, neural tube defect, neuroblastoma, neuronal type of Charcot-Marie-Tooth disease have all been mapped in this region and represent potential targets for a role of NL-2 in humans. NL-3 was localized to chromosome band 2q37. Consistent with a role of NL-3 in bones, a form of Albright hereditary osteodystrophy was mapped to the same chromosomal locus (Phelan et al., 1995).

Production of antibodies against NLs

Antisera collected from injected animals were first tested by immunoblotting on GST-antigen fusion proteins produced in *E. coli*. Antiserum from one rabbit recognized the NL-1-related polypeptide and antisera from one mouse and one rabbit reacted with the NL-3-related polypeptide (results not shown). The anti NL-1 antiserum and the mouse anti NL-3 antiserum, which appeared more specific than the rabbit antiserum, were next tested by immunoblotting on extracts of proteins and culture media from cells expressing NL-1 or NL-3 (see below).

Expression of NL-1 in CHO cells

The cDNA encoding the full-length NL-1 protein was cloned in the mammalian expression vector pcDNA3-RSV and transfected in CHO cells. Stable cell lines were established by selection with the drug G418 and tested by immunoblotting for the presence of NL-1.

Small amounts of NL-1 were found in the extracts of transfected CHO cells (results not shown). This intracellular species was sensitive to endo H digestion,

indicating that the sugar moiety was not mature and suggesting ER localization (results not shown). The culture medium of transfected CHO cells showed the presence of soluble NL-1 (Figure 10). This extracellular species was resistant to endo H suggesting true transport through the late secretory pathway . The cDNA sequence of NL-1 predicts a type II transmembrane protein. The mechanism NL-1 is transformed into a soluble protein is not known presently. However, examination of the amino acid sequence revealed the presence of a putative furin cleavage site from residue 58 to 65 (Figure 3). A similar site is present in NL-2 sequence.

The soluble form of NL-1 was assayed for activity using [^{3}H]-Tyr-(D)Ala$_2$-Leu-enkephalin as substrate. Figure 11 shows that NL-1 can degrade this substrate and that this activity can be inhibited by 10^{-6}M phosphoramidon, a general inhibitor of enzymes of the NEP family.

Use of NL-1 amino-terminal domain to promote secretion

The observation that NL-1 ectodomain was secreted, possibly through cleavage of the transmembrane segment by furin, raised the possibility to promote secretion of exogenous proteins that could be sliced to NL-1 amino-terminal domain (from initiator methionine to the furin site). To test this hypothesis, the ectodomain of NL-3 (from the third cysteine to the end) was spliced to NL-1 amino-terminal domain using a PCR strategy and the recombinant DNA cloned in expression vector pRcCMV. The fusion protein was expressed by transfection of the vector in COS-1 and HEK 293 cells. The culture media of transfected cells was analyzed by immunoblotting using the mouse antiserum against NL-3. Figure 12 shows the presence of NL-3 in the spent culture media of both COS-1 and HEK 293 cells. This result shows that NL-1 amino-terminal domain can be used to promote secretion of exogenous proteins.

The soluble form of NL-3 was assayed for activity using [^{3}H]-Tyr-(D)Ala$_2$-Leu-enkephalin as substrate. No activity was found.

The previous experiment showed that it was possible to use the amino-terminal domain of NL-1 to promote secretion of an otherwise membrane attached protein ectodomain. To verify whether the same strategy could be used to promote secretion of small peptides, a PCR strategy was used to splice human β-endorphin to the amino-terminal domain of NL-1 and the recombinant DNA was cloned in vector pRcCMV. The fusion protein was expressed by transfection of the vector in COS-1 and HEK 293 cells. The culture media of transfected cells was collected 48h after transfection and the peptides purified as described previously (Noël et al., 1989). The presence of β-endorphin in the extracts was detected by radioimmunoassay. The results showed that both COS-1 and HEK 293 cells produced approximately 100 pg of β-endorphin per ml of culture medium.

Cloning of other members of the family

To find other members of the NEP-like family, we will use the same RT-PCR

strategy to amplify mRNA isolated from tissues known to be regulated by peptidergic systems (brain, thymus, kidney, heart, lung, ovary, pancreas, bone, bone marrow and lymphoid cells). In fact, many of these tissues are known to express at least one member of the family and/or to control a peptidergic pathway on which peptidase inhibitors have major effects. Amplified fragments will be cloned and the resulting clones will be sequenced and compared to the sequence of known peptidases, as described above. Pairs of degenerate primers in other highly conserved regions will also be designed to increase the possibility of cloning other relevant peptidases.

DISCUSSION

As discussed above, peptidases of the NEP family known to date have often been found to play important physiological roles. This is certainly true for NEP itself, ECEs and PEX, (see review above). For this reason, some of these enzymes (as it was the case for NEP and ECE in the past) might be interesting targets for the design of inhibitors that in turn could be used as therapeutic agents in various pathological conditions. However, it is of some concern that inhibitors designed for one enzyme may also inhibit to some extent other members of the family. This lack of specificity for an inhibitor used as a therapeutic agent in the long term treatments such as those used as antihypertensive agents for instance, may cause unforeseen problems due to unwanted side effects. The objectives of the present work was to develop a strategy to clone new members of the NEP family of peptidases. The results presented in this report clearly show that our strategy can be successful. We have determined the complete or partial nucleotide sequence of three cDNAs encoding putative enzymes of the NEP family.

These cDNA sequences are valuable tools and may be used to:

Produce antibodies

As shown in the present work, knowledge of NL cDNA sequences can be used to raise specific antibodies. For example but not exclusively, regions of less homology between the peptidases (amino acid residues 50 to 450) can be used to synthesize peptides whose sequences are deduced from the translation of the cDNAs, and/or bacterially-expressed fragments of the cDNAs fused for example but not exclusively to GST may be purified and injected into rabbits or mice for polyclonal or monoclonal antibody production. These antibodies can be used to:

- identify by immunohistochemistry the peptidergic pathways in which the peptidases are functioning;
- study the physiopathology of NL-enzymes by immunoblotting or immunohistochemistry on samples of biological fluids or biopsies;
- set up high through put screening assays to identify NL-enzymes inhibitors. This can be done for example but not exclusively by using the antibodies to attach the NL-enzymes to a solid support;

- purify NL-enzymes with said antibodies by immunoprecipitation or affinity chromatography by identifying antibodies capable of selectively binding to the NL-enzymes in one set of conditions and releasing it in another set of conditions typically involving a large pH or salt concentration change without denaturing the NL-enzyme;
- identify antibodies that block NL-enzymes activities and use them as therapeutic agents. Blocking antibodies can be identified by adding antisera or ascite fluid to an *in vitro* enzymatic assay and looking for inhibition of NL-enzymes activities. Blocking antibodies could then be injected to normal or disease model animals to test for *in vivo* effects.

Derive specific RNA or DNA probes

As shown in the present work, knowledge of the nucleotide sequence of the members of the NEP-family allows nucleotide sequence comparisons and facilitate the design of specific RNA or DNA probes by methods such as but not exclusively molecular cloning, *in vitro* transcription, PCR or DNA synthesis. The probes thus obtained can be used to:

- derive specific probes or oligonucleotides for RNA and DNA analysis, such as Northern blot and *in situ* hybridization, chromosome mapping or PCR testing. These probes could be used for genetic testing of normal or pathological samples of biological fluids or biopsies;
- make vectors for gene knock-out or knock-in in mice. The long range PCR technique and/or screening of a mouse genomic library with probes derived from the 5'-end of the cDNAs can be used to isolate large exon/intron regions. We will then substitute one or more of the cloned genomic DNA exons for the neomycin resistance expression cassette for producing homologous recombination and knock-out mice. Alternatively, cDNAs coding for NLs will be used to overexpressed each of these enzymes in transgenic mice. The cDNAs will be cloned downstream from a promoter sequence, and injected in fertilised mouse eggs. Depending on specific questions to be answered, the chosen promoter sequence will allow expression of the peptidases either in every tissues or in a cell- or tissue-specific manner. Injected eggs will be transferred into foster mothers and the resulting mice analysed for peptidase expression;
- replace defective NL genes in a gene therapy strategy. The NL full length cDNAs could be cloned under the control of a constitutive or inducible promoter having a narrow or wide range of tissue expression and introduced with appropriate vectors in subjects having defective genes;
- synthesise oligonucleotides that could be used to interfere with the expression of the NLs. For example but not exclusively, oligonucleotides with antisens or ribozyme activity could be developed. These oligonucleotides could be

introduced in subjects as described above;

- isolate other members of the family. Screening cDNA and/or genomic libraries with these cDNA probes at low stringency may allow to clone new members of the NEP-like family. Alternatively, alignment of the sequences may allow one to design specific degenerate oligonucleotide primers for RT-PCR screening with mRNA from tissues such as but not exclusively, the hearth and the brain.

Production of recombinant NL-enzymes

As shown in the present work, recombinant active NL-enzymes can be obtained by expression of NL-cDNAs in mammalian cells. From past experience with neprilysin, another member of the family (Devault *et al.*, 1988; Fossiez *et al.*, 1992; Ellefsen *et al.*, submitted), expression can also be performed in other expression systems after cloning of NL-cDNAs in appropriate expression vectors. These expression systems may include but not exclusively the baculovirus/insect cells or larvae system and the *Pichia pastoris*-based yeast system. Production of recombinant NL-enzymes includes the production of naturally occurring membrane bound or soluble forms of the proteins or genetically engineered soluble forms of the enzymes. The latter can be obtained by substituting the cytosolic and trans-membrane domain by a cleavable signal peptide such as that of proopiomelanocortin, but not exclusively, as done previously (Lemay *et al.*, 1989) or by transforming by genetic manipulations the non-cleavable signal peptide membrane anchor domain into a cleavable signal peptide, as done previously (Lemire *et al.*, 1997) or by fusion of the ectodomain of NL-enzymes to the amino-terminal domain (from the initiator methionine to amino acid residue 300) of naturally occurring soluble NLs such as, but not exclusively, NL-1 as done in this work.

These recombinant NLs could be used to:

- find a substrate. A substrate can be identified using one of the following.
  - Screening of existing bioactive peptides. Peptides are incubated in the presence of NL-enzymes and subsequently analysed by HPLC for degradation. Degradation is observed by disappearance of the peak of substrate and the appearance of peaks of products;
  - Screening phage libraries specifically designed for the purpose (phage display library). Each phage expresses at its surface, as part of its coat protein, a random peptide sequence preceded by a peptide sequence recognisable by an antibody or any other sequence-recognizing agent. This latter sequence serves to attach the phage to a solid support. Upon addition of the NL-enzyme the random sequences that are NL substrate are cleaved, releasing the phage. After several rounds of cleavage, the phage sequence is determined to identify the peptide segment recognized by the enzyme.

- Extract of the tissue where the enzyme is expressed is collected and prepared for chromatographic analysis (HPLC, capillary electrophoresis or any other high resolution separation system) by denaturing the extracted proteins with a solvent (acetonitrile or methanol). The extract is subjected to chromatographic separation. The same extract is incubated with the enzyme for a period sufficient to observe a difference between the 2 chromatograms. The regions with the identified changes are collected and subjected to mass spectrometric analysis to determine the peptide compositions.
- Small peptide libraries are prepared with a fluorophore at one extremity and a quencher group at the other (Meldal et al Methods in molecular biology 1998,87). The substrate can be identified using a strategy described in Apletalina et al (JBC (1998)273, 41, 26589-95). For each hexapeptide library, the identity of one residue at one position remains constant while the rest is randomized (for a total of 6*20=120 individual libraries). Each library is made-up of 3.2 million different members and is identified both by the position of the constant residue along the hexapeptide, and its identity. The NL-enzyme is added to each library and the fluorescence is recorded. The data is organized to identify the libraries producing the most fluorescence for each position along the hexapeptide. This arrangement suggests the identity of important residues at each position along the hexapeptide. Hexapeptides representing the best suggestions are prepared and tested in a similar fashion. From this set, the hexapeptide with the best fluorescence is selected.
- set up enzymatic assays. An enzymatic assay consists in the addition of the above identified substrate to the enzyme in constant conditions of pH, salts, temperature and time. The resulting solution is assayed for the hydrolysed peptide or for the intact peptide. This assay can be realized with specific antibodies, HPLC or, when self-quenched fluorescence tagged peptides are used (Meldal et al), by the appearance of fluorescence. The enzyme may be in solution or attached to a solid substrate;
- identify inhibitors. Inhibitors can be identified from synthetic libraries, biota extracts and from rationally designed inhibitors using X-ray crystallography and substituent activity relationships. Each molecule or extract fraction is tested for inhibitory activity using the enzymatic test described above. The molecule responsible for the largest inhibition is further tested to determine its pharmacological and toxicological properties following known procedures. The inhibitor with the best distribution, pharmacological action combined with low toxicity will be selected for drug manufacturing. Pharmaceutically acceptable formulation of the inhibitor or its acceptable salt will be prepared by mixing with known excipients to produce tablets, capsules or injectable solutions. Between

1 and 500mg of the drug is administered to the patients;

- inject the native or soluble purified NL-enzymes into subjects. In the case of disease or pathologies caused by a lack or decrease in NL activity, the purified NL could be injected intravenously or otherwise in patients. Alternatively, immobilized NL-enzymes could be introduced at the site of orthopedic surgery or implantation of devices in bones or dental tissues.

Secretion of foreign proteins and peptides

As shown in the present work, the amino-terminal domain of NL-1 (from the initiator methionine to the furin site) can be used to promote the secretion of a foreign protein (in this case the ectodomain of NL-3 and β-endorphin).

The amino-terminal domain of NL-1 but also of other naturally occurring soluble NL-enzymes could be used to:

- promote production and secretion of foreign proteins. This can be achieved by genetically fusing sequences coding for said foreign proteins downstream from and in phase with the amino-terminal of NL-1. These chimeric constructs could be introduced with the help of appropriate vectors in any of the expression systems mentioned above for protein production and secretion;
- promote production and secretion of bioactive peptides. Sequences encoding small bioactive peptides such as but not exclusively β-endorphin, the enkephalins, substance P, atrial natriuretic peptide (ANF) and osteostatine, could be fused immediately downstream and in phase the furin site of NL-1. These DNA constructs could be used as described above to produce bioactive peptides.
- serve as model to design artificial (non-naturally occurring) proteins or protein segments (protein vectors) to promote secretion of proteins or peptides. These protein vectors can be constructed to resemble a secreted protein. In this case they would be assembled of an endoplasmic reticulum signal peptide, a spacer of varying length and a furin cleavage site to which the protein or peptide destined for secretion can be fused. The total length of the spacer, furin cleavage site and protein or peptide destined for secretion must be at least 70 amino acid residues. Alternatively, such protein vectors could be assembled to resemble a type II membrane protein. In this case they would comprise from the amino to the carboxy-terminus a cytosolic domain of varying length, a transmembrane domain that also acts as a signal peptide, an extracellular segment of varying length and a furin cleavage site to which the protein or peptide destined for secretion can be fused. The total length of the extracellular segment, furin cleavage site and protein or peptide destined for secretion must be at least 70 amino acid residues.

Therapeutic applications of NL-enzymes

The inappropriate processing of endogenous peptides causes several diseases. The inappropriate processing may result from pathologic concentration of the enzyme itself, its substrate or other elements of the biochemical machinery downstream from the controlling enzyme. In this context it is possible to help the patient by managing the activity of the controlling enzyme.

- NL-enzymes have been localized to the brain and may be involved in the improper processing of β-amyloid precursor. Inhibitions of this process by drugs prepared as above, will help patients with Alzheimer disease as well as other patient suffering from diseases caused by plaque formation;
- NL-enzymes may be involved in the improper processing of other peptides involved in neurological diseases, pain or psychiatric disorders. Appropriately designed inhibitors will help in the management of such diseases;
- NL-1 is found in testis and is associated with spermatozoid maturation. Peptides improperly processed by the enzyme may lead to infertility. The addition of NL-1 ex-vivo to seminal liquid or immature spermatozoids taken directly from testis during an in-vitro fertilization procedure will increase fertility. Conversely, the use of a small-molecule inhibitor or removal of NL-1 with an antibody could increase fertility during an in-vitro fertilization procedure. The administration of a NL-1 inhibitor may increase or decrease the fertility potential. This inhibitor is formulated and administered as described above.
- NL-3 is found in ovaries and may be involved in the processing of a peptide involved in the maturation of eggs. The addition of NL-3 ex-vivo to immature eggs taken directly from ovaries during an in-vitro fertilization procedure will increase fertility. Conversely, the use of a small-molecule inhibitor or removal of NL-3 with an antibody could increase fertility during an in-vitro fertilization procedure. This inhibitor is formulated and administered as described above;
- NL-3 is found in bones. The improper processing of peptides by the enzyme may result in bone disease or abnormal phosphate metabolism. Administration of an inhibitor, as described above, will allow the disease management.

TABLE I

Extend of amino acid sequence identity between members of the NEP-like family

	hNEP	hPEX	hECE-1A	hECE-2	hKELL	sNL-1	hNL-2	hNL-3
hNEP	100*							
hPEX	35	100						
hECE-1A	39	38	100					
hECE-2	36	37	62	100				
hKELL	23	24	30	31	100			

sNL-1	54	39	40	40	25	100		
hNL-2	54	39	39	39	26	77	100	
hNL-3	35	32	37	37	28	36	34	100

*: percentage of sequence identity

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What is claimed is:

1. A neutral endopeptidase-like metallopeptidase named NL-1 which is isolable from testis, which proteic precursor has 765 amino acids, and which shares about 54-65% homology with neutral endopeptidase enzyme, or a variant thereof.

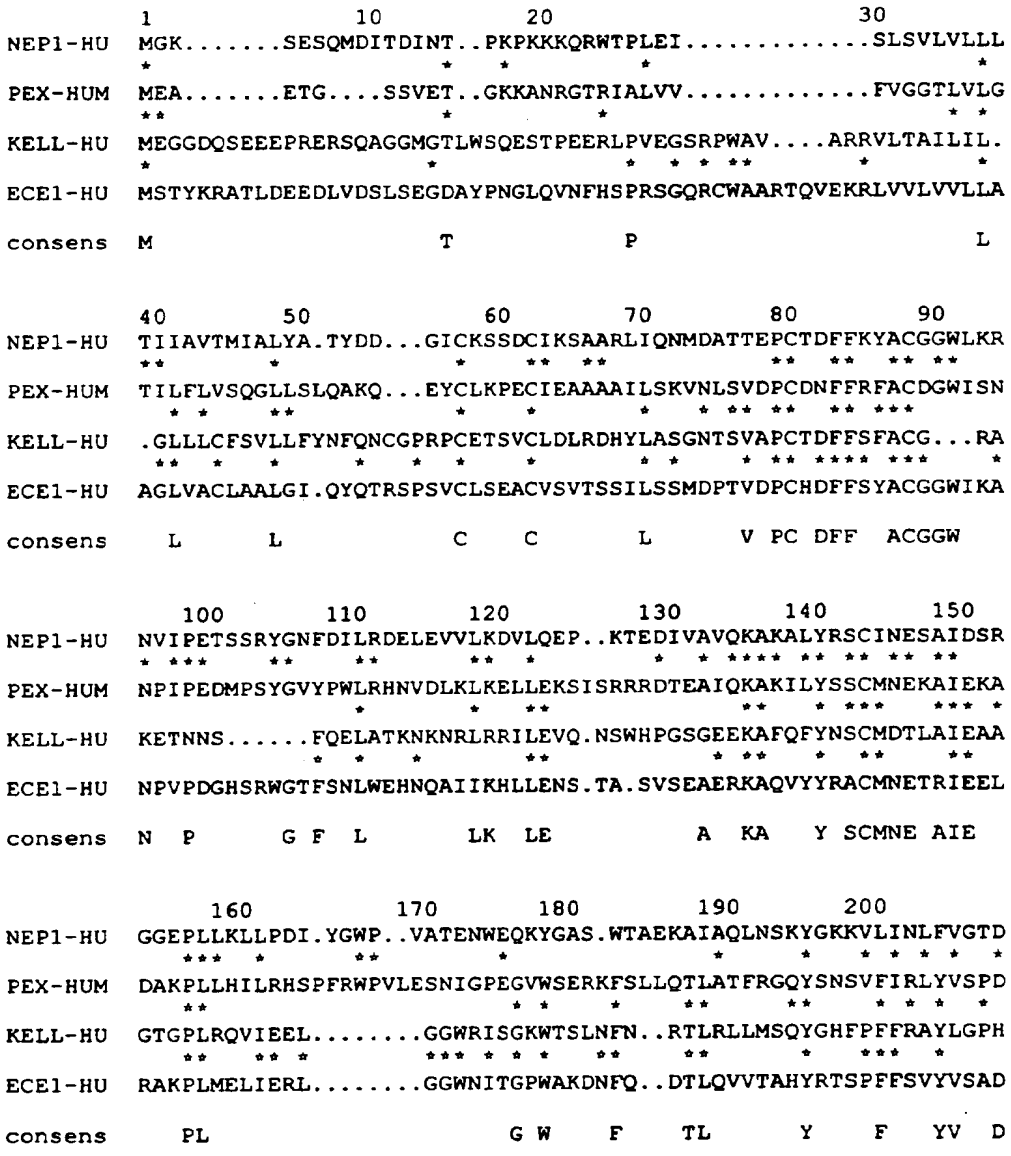
2. A metallopeptidase as defined in claim 1, wherein said proteic precursor has the amino acid sequence shown in Figure 3.

3. A neutral endopeptidase-like metallopeptidase named NL-2, which is isolable from brain, which proteic precursor has 770 amino acids, and which shares about 77% homology with the metallopeptidase defined in claim 1 or 2, or a variant thereof.

4. A metalloprotease as defined in claim 3, wherein said proteic precursor has the amino acid sequence shown in Figure 4.

5. A neutral endopeptidase-like metallopeptidase named NL-3, which is isolable from bone, which proteic precursor comprises the 295 amino acids shown in Figure 5, and which shares about 28 to 37% homology with members of the neutral endopeptidase-like family including the metallopeptidase defined in any one of claims 1 to 4, or a variant thereof.

FIGURE 1


1 10 20 30
NEP1-HU MGK.....SESQMDITDINT..PKPKKKQRWTPLEI.....SLSVLVLLL
PEX-HUM MEA.....ETG....SSVET..GKKANRGTRIALVV.....FVGGTLVLG
KELL-HU MEGGDQSEEEPRERSQAGGMGTLWSQESTPEERLPVEGSRPWAV....ARRVLTAILIL.
ECE1-HU MSTYKRATLDEEDLVDSLSEGDAYPNGLQVNFHSPRSGQRCWAARTQVEKRLVVLVVLLA
consens M T P L
40 50 60 70 80 90
NEP1-HU TIIAVTMIALYA.TYDD...GICKSSDCIKSAARLIQNMDATTEPCTDFFKYACGGWLKR
PEX-HUM TILFLVSQGLLSLQAKQ...EYCLKPECIEAAAAILSKVNLSVDPCDNFFRFACDGWISN
KELL-HU .GLLLCFSVLLFYNFQNCGPRPCETSVCLDLRDHYLASGNTSVAPCTDFFSFACG...RA
ECE1-HU AGLVACLAALGI.QYQTRSPSVCLSEACVSVTSSILSSMDPTVDPCHDFFSYACGGWIKA
consens L L C C L V PC DFF ACGGW
100 110 120 130 140 150
NEP1-HU NVIPETSSRYGNFDILRDELEVVLKDVLQEP..KTEDIVAVQKAKALYRSCINESAIDSR
PEX-HUM NPIPEDMPSYGVYPWLRHNVDLKLKELLEKSISRRRDTEAIQKAKILYSSCMNEKAIEKA
KELL-HU KETNNS.....FQELATKNKNRLRRILEVQ.NSWHPGSGEEKAFQFYNSCMDTLAIEAA
ECE1-HU NPVPDGHSRWGTFSNLWEHNQAIIKHLLENS.TA.SVSEAERKAQVYYRACMNETRIEEL
consens N P G F L LK LE A KA Y SCMNE AIE
160 170 180 190 200
NEP1-HU GGEPLLKLLPDI.YGWP..VATENWEQKYGAS.WTAEKAIAQLNSKYGKKVLINLFVGTD
PEX-HUM DAKPLLHILRHSPFRWPVLESNIGPEGVWSERKFSLLQTLATFRGQYSNSVFIRLYVSPD
KELL-HU GTGPLRQVIEEL.....GGWRISGKWTSLNFN..RTLRLLMSQYGHFPFFRAYLGPH
ECE1-HU RAKPLMELIERL.....GGWNITGPWAKDNFQ..DTLQVVTAHYRTSPFFSVYVSAD
consens PL G W F TL Y F YV D

```
                  220        230        240        250        260
NEP1-HU  DKNSVNHVIHIDQPRLGLPSR.DYYECTGIYKEACTAYVDFMISVARLIRQEERLPI.DE
         ** *  *    **  * *  * **       *    *   **   * *       *
PEX-HUM  DKASNEHILKLDQATLSLAVREDYLDNSTEAKSYRDALYKFMVDTAVL.......LGA.NS
                   **          *    *           *       **
KELL-HU  PASPHTPVIQIDQPEFDVPLKQDQEQKI.YAQIFRE.YLTYLNQLGTL.......LGG.DP
          *** **     *        *           ** *  *** *        *** *
ECE1-HU  SKNSNSNVIQVDQSGLGLPSRDYYLNKTENEKVLTG.YLNYMVQLGKL.......LGGGDE

consens   K S   VI  DQ  L LP R DY       K     Y   M     L       LG  D

          270        280        290        300        310        320
NEP1-HU  NQLALEMNKVMELEKEIANATAKPEDRNDPMLLYNKMTLAQIQNNFSLEINGKPFSWLNF
         *  *  **  **       * *        ***                    * **
PEX-HUM  SRAEHDMKSVLRLEIKIAEIMIPHENRTSEAMY.NKMNISELSAMIP.......QFDWLGY
*                    * * *              *  *  * *        ***
KELL-HU  SKVQEHSSLSISITSRLFQFLRPLEQRRAQGKLFQMVTIDQLKEMAP.......AIDWLSC
                     *    * * **         **   *   **       ** **
ECE1-HU  EAIRPQMQQILDFETALANITIPQEKRRDEELIYHKVTAAELQTLAP.......AINWLPF

consens        M      E   A    P E R        K T   L    P         WL

          330        340           350        360        370        380
NEP1-HU  TNEIMSTVNISITNEEDVVVYA....PEYLTKLKPILTKYSARDLQNLMSWRFIMDLVSS
         *                        * *   *  **          *   **
PEX-HUM  IKKVIDTRLYPHLKDISPSENVVVRVPQYFKDLFRILGSERKKTIANYLVWRMVYSRIPN
                                        *                    *    *
KELL-HU  LQATFTPMSLSPSQSLVVHDVEYL...KNMSQLVEEMLLKQRDFLQSHMILGLVVTLSPA
*   * *      *   ** * ***       * *          *  *   **  **   *
ECE1-HU  LNTIFYPVEINESEPIVVYDKEYL...EQISTLINT...TDRCLLNNYMIWNLVRKTSSF

consens                    V              L           L N M W  V

               390           400        410        420        430
NEP1-HU  LSRTYKESR....NAFRKALYGTT.SETATWRRCANYVNGNMENAVGRLYVEAAFAGESK
         ***          *    ***       *  * *          **   *   *    *
PEX-HUM  LSRRFQYRW....LEFSRVIQGTT.TLLPQWDKCVNFIESALPYVVGKMFVDVYFQEDKK
         *   **                        * * ***              **   *
KELL-HU  LDSQFQEARRKLSQKLRELTEQPPMPARPRWMKCVEETGTFFEPTLAALFVREAFGPSTR
         **  ** *      *  *          ***  **  *        *   **   *
ECE1-HU  LDQRFQDA....DEKFMEVMYGTKKTCLPRWKFCVSDTENNLGFALGPMFVKATFAEDSK

consens  L   FQ         F     GT     P W  CV           G  FV   F    K
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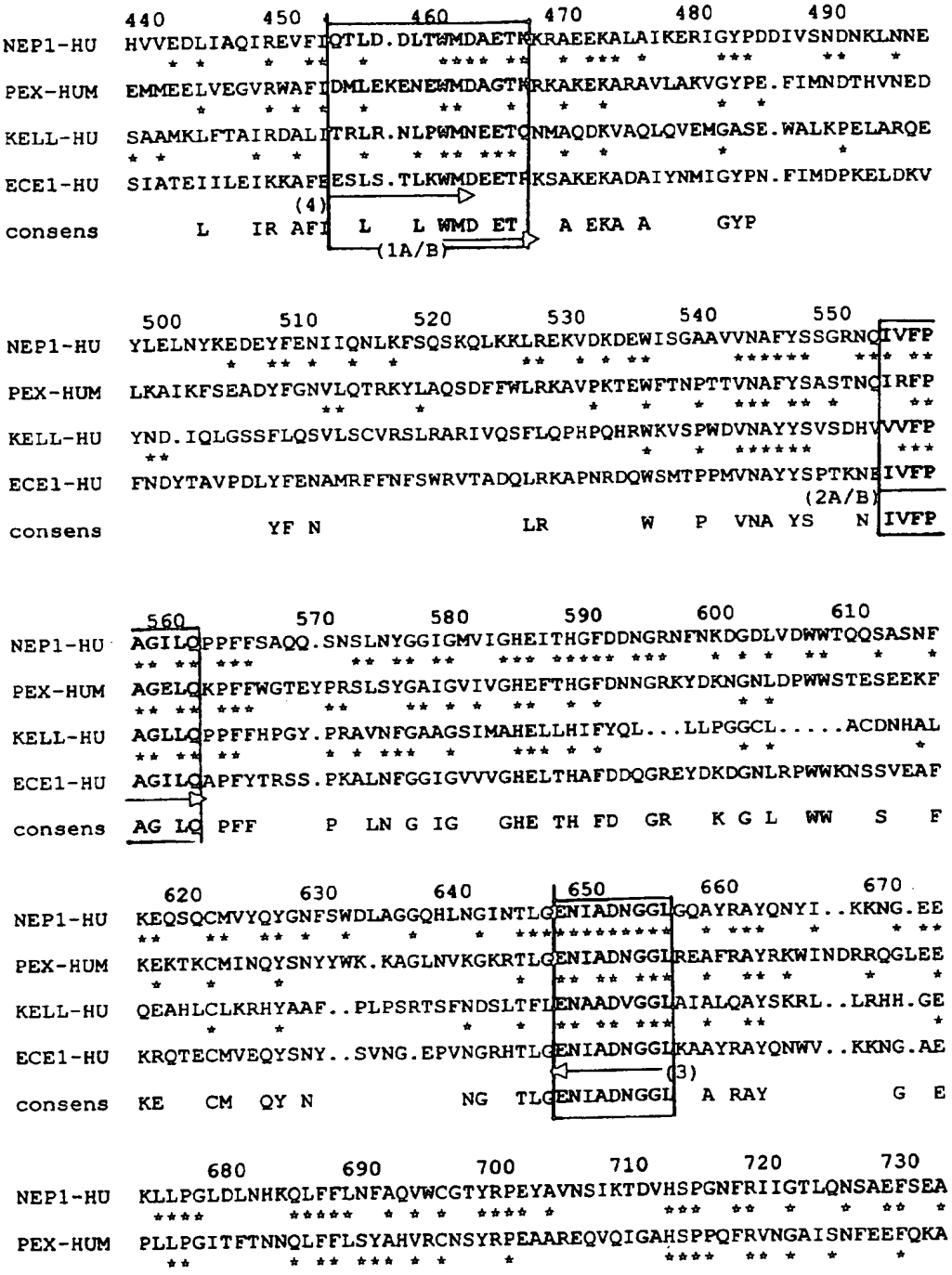
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NEP1-HU HVVEDLIAQIREVFIQTLD.DLTWMDAETKKRAEEKALAIKERIGYPDDIVSNDNKLNNE
PEX-HUM EMMEELVEGVRWAFIDMLEKENEWMDAGTKRKAKEKARAVLAKVGYPE.FIMNDTHVNED
KELL-HU SAAMKLFTAIRDALITRLR.NLPWMNEETQNMAQDKVAQLQVEMGASE.WALKPELARQE
ECE1-HU SIATEIILEIKKAFEESLS.TLKWMDEETRKSAKEKADAIYNMIGYPN.FIMDPKELDKV
(4)
consens L IR AFI L L WMD ET A EKA A GYP
(1A/B)
500 510 520 530 540 550
NEP1-HU YLELNYKEDEYFENIIQNLKFSQSKQLKKLREKVDKDEWISGAAVVNAFYSSGRNQIVFP
PEX-HUM LKAIKFSEADYFGNVLQTRKYLAQSDFFWLRKAVPKTEWFTNPTTVNAFYSASTNQIRFP
KELL-HU YND.IQLGSSFLQSVLSCVRSLRARIVQSFLQPHPQHRWKVSPWDVNAYYSVSDHVVFP
ECE1-HU FNDYTAVPDLYFENAMRFFNFSWRVTADQLRKAPNRDQWSMTPPMVNAYYSPTKNEIVFP
(2A/B)
consens YF N LR W P VNA YS N IVFP
560 570 580 590 600 610
NEP1-HU AGILQPPFFSAQQ.SNSLNYGGIGMVIGHEITHGFDDNGRNFNKDGDLVDWWTQQSASNF
PEX-HUM AGELQKPFFWGTEYPRSLSYGAIGVIVGHEFTHGFDNNGRKYDKNGNLDPWWSTESEEKF
KELL-HU AGLLQPPFFHPGY.PRAVNFGAAGSIMAHELLHIFYQL...LLPGGCL.....ACDNHAL
ECE1-HU AGILQAPFYTRSS.PKALNFGGIGVVVGHELTHAFDDQGREYDKDGNLRPWWKNSSVEAF
consens AG LQ PFF P LN G IG GHE TH FD GR K G L WW S F
620 630 640 650 660 670
NEP1-HU KEQSQCMVYQYGNFSWDLAGGQHLNGINTLGENIADNGGLGQAYRAYQNYI..KKNG.EE
PEX-HUM KEKTKCMINQYSNYYWK.KAGLNVKGKRTLGENIADNGGLREAFRAYRKWINDRRQGLEE
KELL-HU QEAHLCLKRHYAAF..PLPSRTSFNDSLTFLENAADVGGLAIALQAYSKRL..LRHH.GE
ECE1-HU KRQTECMVEQYSNY..SVNG.EPVNGRHTLGENIADNGGLKAAYRAYQNWV..KKNG.AE
(3)
consens KE CM QY N NG TLGENIADNGGL A RAY G E
680 690 700 710 720 730
NEP1-HU KLLPGLDLNHKQLFFLNFAQVWCGTYRPEYAVNSIKTDVHSPGNFRIIGTLQNSAEFSEA
PEX-HUM PLLPGITFTNNQLFFLSYAHVRCNSYRPEAAREQVQIGAHSPPQFRVNGAISNFEEFQKA

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KELL-HU   TVLPSLDLSPQQIFFRSYAQVMCRKPSPQDSH.....DTHSPPHLRVHGPLSSTPAFARY
ECE1-HU   HSLPTLGLTNNQLFFLGFAQVWCSVRTPESSHEGLITDPHSPSRFRVIGSLSNSKEFSEH
consens     LP L L   QLFFL  AQV C    PE        D HSP  FRV G LSN  EF
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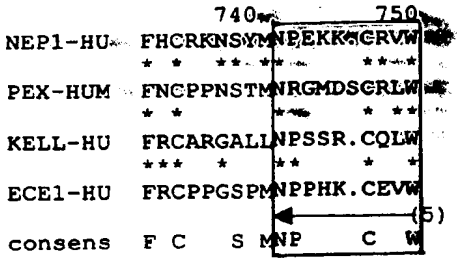


FIGURE 2

PRIMER	SEQUENCE
(1A)	5'-TGGATGGAT/CGA/CIGG/AIACIA/CA-3'
(1B)	5'-TGGATGGAT/CGA/CIGG/AIACIA/CG-3'
(2A)	5'-A/GTIGTITTT/CCCIGCIGGIA/GT/AIC/TTA/TCA-3'
(2B)	5'-A/GTIGTITTT/CCCIGCIGGIA/GT/AIC/TTG/CCA-3'
(3)	5'-AIICCICCIA/TC/TA/GTCIGCIG/AC/TA/GTTT/CTC-3'
(4)	5'-GAT/CAAT/CT/CTIGAT/CGAA/GT/CTIAAT/CTGGATGG-3'
(5)	5'-T/CT/CACCAIATICT/GA/GCATCG/TT/CTTCATIGGG/ATG-3'

FIGURE 3

Sequence of NL-1 cDNA from mouse

```
                                                                                                                  30
    met val glu arg ala gly trp cys arg lys lys ser pro gly phe val glu tyr gly leu met val leu leu leu leu leu gly ala
  1 ATG GTG GAG AGA GCA GGC TGG TGT CGG AAG AAG TCC CCA GGC TTC GTG GAG TAT GGG CTG ATG GTG CTG CTG CTG CTG TTG CTG GGA GCC
                                                                                                                  60
    ile val thr leu gly val phe tyr ser ile gly lys gln leu pro leu leu thr ser leu leu his phe ser trp asp glu arg thr val
 91 ATA GTG ACT CTG GGT GTC TTC TAC AGC ATA GGG AAG CAG CTG CCC CTC TTA ACT AGC CTG CTA CAC TTC TCC TGG GAT GAG AGG ACG GTT
                                                                                                                  90
    val lys arg ala leu arg asp ser ser leu lys ser asp ile cys thr thr pro ser cys val ile ala ala ala arg ile leu glu asn
181 GTA AAA CGA GCC CTC AGG GAT TCA TCA CTG AAA AGT GAC ATC TGC ACC ACC CCA AGC TGT GTG ATA GCA GCT GCC AGA ATC CTC GAA AAC
                                                                                                                 120
    met asp gln ser arg asn pro cys glu asn phe tyr gln tyr ala cys gly gly trp leu arg his his val ile pro glu thr asn ser
271 ATG GAC CAA TCG AGG AAC CCC TGT GAA AAC TTC TAC CAG TAC GCC TGC GGA GGC TGG CTG AGG CAC CAC GTG ATC CCA GAG ACC AAC TCC
                                                                                                                 150
    arg tyr ser val phe asp ile leu arg asp glu leu glu val ile leu lys gly val leu glu asp ser thr ser gln his arg pro ala
361 CGA TAC AGC GTC TTT GAC ATC CTG CGG GAC GAG CTG GAG GTT ATC CTC AAA GGG GTG CTG GAG GAT TCC ACT TCC CAG CAT CGC CCG GCC
                                                                                                                 180
    val glu lys ala lys thr leu tyr arg ser cys met asn gln ser val ile glu lys arg asp ser glu pro leu leu ser val leu lys
451 GTG GAG AAG GCC AAG ACA CTA TAT CGC TCC TGC ATG AAC CAA AGT GTG ATC GAG AAG AGA GAC TCT GAG CCC CTG CTG AGC GTC TTA AAA
                                                                                                                 210
    met val gly gly trp pro val ala met asp lys trp asn glu thr met gly leu lys trp glu leu glu arg gln leu ala val leu asn
541 ATG GTA GGA GGT TGG CCT GTG GCC ATG GAT AAG TGG AAC GAG ACC ATG GGC CTC AAG TGG GAA CTG GAG CGA CAG TTG GCT GTG TTG AAC
                                                                                                                 240
    ser gln phe asn arg arg val leu ile asp leu phe ile trp asn asp asp gln asn ser ser arg his val ile tyr ile asp gln pro
631 TCG CAG TTC AAC AGG CGG GTC CTC ATC GAC CTC TTC ATC TGG AAT GAC GAC CAG AAC TCC AGC CGG CAT GTC ATC TAC ATA GAC CAG CCC
                                                                                                                 270
    thr leu gly met pro ser arg glu tyr tyr phe gln glu asp asn asn his lys val arg lys ala tyr leu glu phe met thr ser val
721 ACC TTG GGC ATG CCA TCC CGG GAG TAC TAT TTC CAG GAG GAC AAC AAC CAC AAG GTA CGG AAA GCC TAC CTG GAG TTC ATG ACG TCA GTG
                                                                                                                 300
    ala thr met leu arg lys asp gln asn leu ser lys glu ser ala met val arg glu glu met ala glu val leu glu leu glu thr his
811 GCC ACT ATG CTT AGG AAA GAC CAG AAC CTG TCC AAG GAG AGC GCC ATG GTG CGG GAG GAG ATG GCG GAG GTG CTG GAA CTG GAG ACG CAT
                                                                                                                 330
    leu ala asn ala thr val pro gln glu lys arg his asp val thr ala leu tyr his arg met asp leu met glu leu gln glu arg phe
901 CTG GCC AAC GCC ACA GTC CCC CAG GAG AAA AGG CAT GAT GTC ACT GCC CTG TAC CAC CGA ATG GAC CTG ATG GAG CTA CAG GAA AGG TTT
                                                                                                                 360
    gly leu lys gly phe asn trp thr leu phe ile gln asn val leu ser ser val glu val glu leu phe pro asp glu glu val val val
991 GGT CTG AAG GGG TTT AAC TGG ACT CTC TTC ATA CAA AAC GTG TTG TCT TCT GTG GAA GTC GAG CTG TTC CCA GAT GAG GAG GTG GTG GTC
                                                                                                                 390
    tyr gly ile pro tyr leu glu asn leu glu asp ile ile asp ser tyr ser ala arg thr met gln asn tyr leu val trp arg leu val
1081 TAC GGC ATC CCC TAC CTG GAG AAT CTG GAG GAT ATC ATT GAT AGC TAC TCA GCA CGG ACC ATG CAG AAC TAC CTG GTA TGG CGC CTG GTG
                                                                                                                 420
    leu asp arg ile gly ser leu ser gln arg phe lys glu ala arg val asp tyr arg lys ala leu tyr gly thr thr val glu glu val
1171 CTA GAT CGA ATT GGC AGC CTG AGC CAG AGA TTC AAA GAG GCG CGT GTG GAC TAC CGC AAG GCG CTG TAC GGC ACG ACC GTG GAG GAG GTA
                                                                                                                 450
```

```
    arg trp arg glu cys val ser tyr val asn ser asn met glu ser ala val gly ser leu tyr ile lys arg ala phe ser lys asp ser
1261 CGC TGG CGA GAG TGT GTC AGC TAT GTC AAC AGT AAC ATG GAG AGC GCC GTG GGC TCC CTC TAC ATC AAG CGG GCC TTC TCC AAG GAC AGC
                                                                                                                    480

    lys ser thr val arg glu leu ile glu lys ile arg ser val phe val asp asn leu asp glu leu asn trp met asp glu glu ser lys
1351 AAG AGC ACG GTC AGA GAG CTG ATT GAG AAG ATA AGG TCC GTG TTT GTG GAT AAC CTG GAT GAG CTG AAC TGG ATG GAC GAG GAA TCC AAG
                                                                                                                    510

    lys lys ala gln glu lys ala met asn ile arg glu gln ile gly tyr pro asp tyr ile leu glu asp asn asn lys his leu asp glu
1441 AAG AAG GCC CAG GAA AAG GCC ATG AAT ATA CGG GAA CAG ATT GGC TAC CCT GAC TAC ATT TTG GAA GAT AAC AAT AAA CAC CTG GAT GAG
                                                                                                                    540

    glu tyr ser ser leu thr phe tyr glu asp leu tyr phe glu asn gly leu gln asn leu lys asn asn ala gln arg ser leu lys lys
1531 GAA TAC TCC AGT TTG ACT TTC TAT GAG GAC CTG TAT TTT GAG AAC GGA CTT CAG AAC CTC AAG AAC AAT GCC CAG AGG AGC CTC AAG AAG
                                                                                                                    570

    leu arg glu lys val asp gln asn leu trp ile ile gly ala ala val val asn ala phe tyr ser pro asn arg asn gln ile val phe
1621 CTT CGG GAA AAG GTG GAC CAG AAT CTC TGG ATC ATC GGG GCT GCA GTG GTC AAT GCA TTC TAC TCC CCA AAC AGA AAC CAG ATC GTC TTT
                                                                                                                    600

    pro ala gly ile leu gln pro pro phe phe ser lys asp gln pro gln ser leu asn phe gly gly ile gly met val ile gly his glu
1711 CCA GCA GGG ATT CTC CAG CCG CCC TTC TTC AGC AAG GAC CAA CCA CAG TCC TTG AAT TTT GGG GGC ATC GGG ATG GTG ATT GGG CAC GAG
                                                                                                                    630

    ile thr his gly phe asp asp asn gly arg asn phe asp lys asn gly asn met leu asp trp trp ser asn phe ser ala arg his phe
1801 ATC ACA CAC GGC TTT GAT GAT AAT GGT CGT AAC TTT GAC AAG AAC GGC AAC ATG CTG GAC TGG TGG AGT AAC TTC TCG GCC CGG CAC TTC
                                                                                                                    660

    gln gln gln ser gln cys met ile tyr gln tyr gly asn phe ser trp glu leu ala asp asn gln asn val asn gly phe ser thr leu
1891 CAA CAG CAG TCG CAA TGC ATG ATC TAT CAG TAC GGC AAC TTC TCT TGG GAA CTA GCA GAC AAC CAG AAT GTG AAC GGA TTC AGT ACC CTC
                                                                                                                    690

    gly glu asn ile ala asp asn gly gly val arg gln ala tyr lys ala tyr leu arg trp leu ala asp gly gly lys asp gln arg leu
1981 GGG GAG AAC ATT GCC GAC AAC GGA GGT GTG CGA CAG GCA TAC AAG GCT TAC CTA CGG TGG CTG GCT GAT GGC GGC AAA GAT CAG CGA CTG
                                                                                                                    720

    pro gly leu asn leu thr tyr ala gln leu phe phe ile asn tyr ala gln val trp cys gly ser tyr arg pro glu phe ala val gln
2071 CCG GGA CTG AAC CTG ACC TAT GCC CAG CTT TTC TTC ATC AAC TAT GCC CAG GTG TGG TGT GGG TCC TAT AGG CCG GAG TTC GCC GTC CAG
                                                                                                                    750

    ser ile lys thr asp val his ser pro leu lys tyr arg val leu gly ser leu gln asn leu pro gly phe ser glu ala phe his cys
2161 TCC ATC AAG ACG GAC GTC CAC AGT CCT CTT AAG TAC AGG GTG CTG GGC TCA CTA CAG AAC CTG CCA GGC TTC TCT GAG GCA TTC CAC TGC
                                                                                                                    765

    pro arg gly ser pro met his pro met lys arg cys arg ile trp ***
2251 CCA CGA GGC AGC CCC ATG CAC CCC ATG AAG CGA TGT CGC ATC TGG TAG CCAAGGCTGAGCTATGCTGCGGCCCACGCCCCGCCACCCAGAGGCTTCGCGAATG

2354 GTGTAGCTGGCAGAGATGTGCAGGTCTTTGCCTGAAGGCCACCGGAGCCACCAGCCAGCCCTCCGCGCCCAGCCTAGAGTGTAGCCACCCGCCCACACCCGGGATGAGTGGTGCCGGTC

2473 CTGCGCCCCCTCAGGCCAGTGAGGGTCAGCAGCCCAGGAAGAGCAGTCAGCTGCCTTCCACCCTCTCCATAGTGTGTGGCTAAATGTTCTCGAGCTTCAGACTTGAGCTAAGTAAACGC

2592 T
```

FIGURE 4

Partial sequence OF NL-2 cDNA from human

```
                                                                                                                                30
   arg gly ile pro glu ala gln glu val ser glu val cys thr thr pro gly cys val ile ala ala ala arg ile leu gln asn met asp
 1 CGG GGG ATC CCA GAG GCC CAG GAG GTG AGC GAG GTC TGC ACC ACC CCT GGC TGC GTG ATA GCA GCC GCC AGG ATC CTC CAG AAC ATG GAC
                                                                                                                                60
   pro thr thr glu pro cys asp asp phe tyr gln phe ala cys gly gly trp leu arg arg his val ile pro glu thr asn ser arg tyr
91 CCG ACC ACG GAA CCG TGT GAC GAC TTC TAC CAG TTT GCA TGC GGA GGC TGG CTG CGG CGC CAC GTG ATC CCT GAG ACC AAC TCA AGA TAC
                                                                                                                                90
   ser ile phe asp val leu arg asp glu leu glu val ile leu lys ala val leu glu asn ser thr ala lys asp arg pro ala val glu
181 AGC ATC TTT GAC GTC CTC CGC GAC GAG CTG GAG GTC ATC CTC AAA GCG GTG CTG GAG AAT TCG ACT GCC AAG GAC CGG CCG GCT GTG GAG
                                                                                                                               120
   lys ala arg thr leu tyr arg ser cys met asn gln ser val ile glu lys arg gly ser gln pro leu leu asp ile leu glu val val
271 AAG GCC AGG ACG CTG TAC CGC TCC TGC ATG AAC CAG AGT GTG ATA GAG AAG CGA GGC TCT CAG CCC CTG CTG GAC ATC TTG GAG GTG GTG
                                                                                                                               150
   ter gly trp pro val ala met asp arg trp asn glu thr val gly leu glu trp glu leu glu arg his leu ala leu met asn ser gln
361 TGA GGC TGG CCG GTG GCG ATG GAC AGG TGG AAC GAG ACC GTA GGA CTC GAG TGG GAG TTG GAG CGG CAC CTG GCG CTG ATG AAC TCA CAG
                                                                                                                               180
   phe asn arg arg val leu ile asp leu phe ile trp asn asp asp gln asn ser ser arg his ile ile tyr ile asp gln pro thr leu
451 TTC AAC AGG CGC GTC CTC ATC GAC CTC TTC ATC TGG AAC GAC GAC CAG AAC TCC AGC CGG CAC ATC ATC TAC ATA GAC CAG CCC ACC TTG
                                                                                                                               210
   gly met pro ser arg glu tyr tyr phe asn his gly ser asn arg lys val arg glu ala tyr leu gln phe met val ser val ala thr
541 GGC ATG CCC TCC CGA GAG TAC TAC TTC AAC CAC GGC AGC AAG GGG AAG GTG CGG GAA GCC TAC CTG CAG TTC ATG GTG TCA GTG GCC ACG
                                                                                                                               240
   leu leu arg glu asp ala asn leu pro arg asp ser cys leu val gln glu asp met val gln val leu glu leu glu thr gln leu ala
631 TTG CTG CGG GAG GAT GCA AAC CTG CCC AGG GAC AGC TGC CTG GTG CAG GAG GAC ATG GTG CAG GTT CTG GAG CTG GAG ACA CAG CTG GCC
                                                                                                                               270
   lys ala thr val pro gln glu glu arg his asp val ile ala leu tyr his arg met gly leu glu glu leu gln ser gln phe gly leu
721 AAG GCC ACG GTA CCC CAG GAG GAG AGA CAC GAC GTC ATC GCC TTG TAC CAC CGG ATG GGA CTG GAG GAG CTG CAA AGC CAG TTT GGC CTG
                                                                                                                               300
   lys gly phe asn trp thr leu phe ile gln thr val leu ser ser val lys ile lys leu leu pro asp glu glu val val val tyr gly
811 AAG GGA TTT AAC TGG ACT CTG TTC ATA CAA ACT GTG CTA TCC TCT GTC AAA ATC AAG CTG CTG CCA GAT GAG GAA GTG GTG GTC TAT GGC
                                                                                                                               330
   ile pro tyr leu gln asn leu glu asn ile ile asp thr tyr ser ala arg thr ile gln asn tyr leu val trp arg leu val leu asp
901 ATC CCC TAC CTG CAG AAC CTT GAA AAC ATC ATC GAC ACC TAC TCA GCC AGG ACC ATA CAG AAC TAC CTG GTC TGG CGC CTG GTG CTG GAC
                                                                                                                               360
   arg ile gly ser leu ser gln arg phe lys asp thr arg val asn tyr arg lys ala leu phe gly thr met val glu glu val arg trp
991 CGC ATT GGT AGC CTA AGC CAG AGA TTC AAG GAC ACA CGA GTG AAC TAC CGC AAG GCG CTG TTT GGC ACA ATG GTG GAG GAG GTG CGC TGG
                                                                                                                               390
```

```
     arg glu cys val gly tyr val asn ser asn met glu asn ala val gly ser leu tyr val arg glu ala phe pro gly asp ser lys ser
1081 CGT GAA TGT GTG GGC TAC GTC AAC AGC AAC ATG GAG AAC GCC GTG GGC TCC CTC TAC GTC AGG GAG GCG TTC CCT GGA GAC AGC AAG AGC
                                                                                                                      420
     met val arg glu leu ile asp lys val arg thr val phe val glu thr leu asp glu leu gly trp met asp glu glu ser lys lys lys
1171 ATG GTC AGA GAA CTC ATT GAC AAG GTG CGG ACA GTG TTT GTG GAG ACG CTG GAC GAG CTG GGC TGG ATG GAC GAG GAG TCC AAG AAG AAG
                                                                                                                      450
     val gln glu lys ala met ser ile arg glu gln ile gly his arg asp tyr ile leu glu glu thr asn arg arg leu asp glu gly tyr
1261 GTG CAG GAG AAG GCC ATG AGC ATC CGG GAG CAG ATC GGG CAC CGT GAC TAC ATC CTG GAG GAG ACG AAC AGG CGC CTG GAC GAG GGG TAC
                                                                                                                      480
     ser asn leu asn phe ser glu asp leu tyr phe glu asn ser leu gln asn leu lys val gly ala gln arg ser leu arg lys leu arg
1351 TCC AAT CTG AAC TTC TCA GAG GAC CTG TAC TTT GAG AAC AGT CTG CAG AAC CTC AAG GTG GGC GCC CAG CGG AGC CTC AGG AAG CTT CGG
                                                                                                                      510
     glu lys val asp pro asn leu trp ile ile gly ala ala val val asn ala phe tyr ser pro asn arg asn gln ile val phe pro ala
1441 GAA AAG GTG GAC CCA AAT CTC TGG ATC ATC GGG GCG GCG GTG GTC AAT GCG TTC TAC TCC CCA AAC CGA AAC CAG ATT GTA TTC CCT GCC
                                                                                                                      540
     gly ile leu gln pro pro phe phe ser lys glu gln pro gln ala leu asn phe gly gly ile gly met val ile gly his glu ile thr
1531 GGG ATC CTC CAG CCC CCC TTC TTC AGC AAG GAG CAG CCA CAG GCC TTG AAC TTT GGA GGC ATT GGG ATG GTG ATC GGG CAC GAG ATC ACG
                                                                                                                      570
     his gly phe asp asp asn gly arg asn phe asp lys asn gly asn met met asp trp trp ser asn phe ser thr gln his phe arg glu
1621 CAC GGC TTT GAC GAC AAT GGC CGG AAC TTC GAC AAG AAT GGC AAC ATG ATG GAT TGG TGG AGT AAC TTC TCC ACC CAG CAC TTC CGG GAG
                                                                                                                      600
     gln ser glu cys met ile tyr gln tyr gly asn tyr ser trp asp leu ala asp gly gln asn val asn gly phe asn thr leu gly glu
1711 CAG TCA GAG TGC ATG ATC TAC CAG TAC GGC AAC TAC TCC TGG GAC CTG GCA GAC GGA CAG AAC GTG AAC GGA TTC AAC ACC CTT GGG GAA
                                       609
     asn ile ala asp asn gly ala gly ile
1801 AAC ATT GCT GAC AAC GGA GCC GGA ATT
```

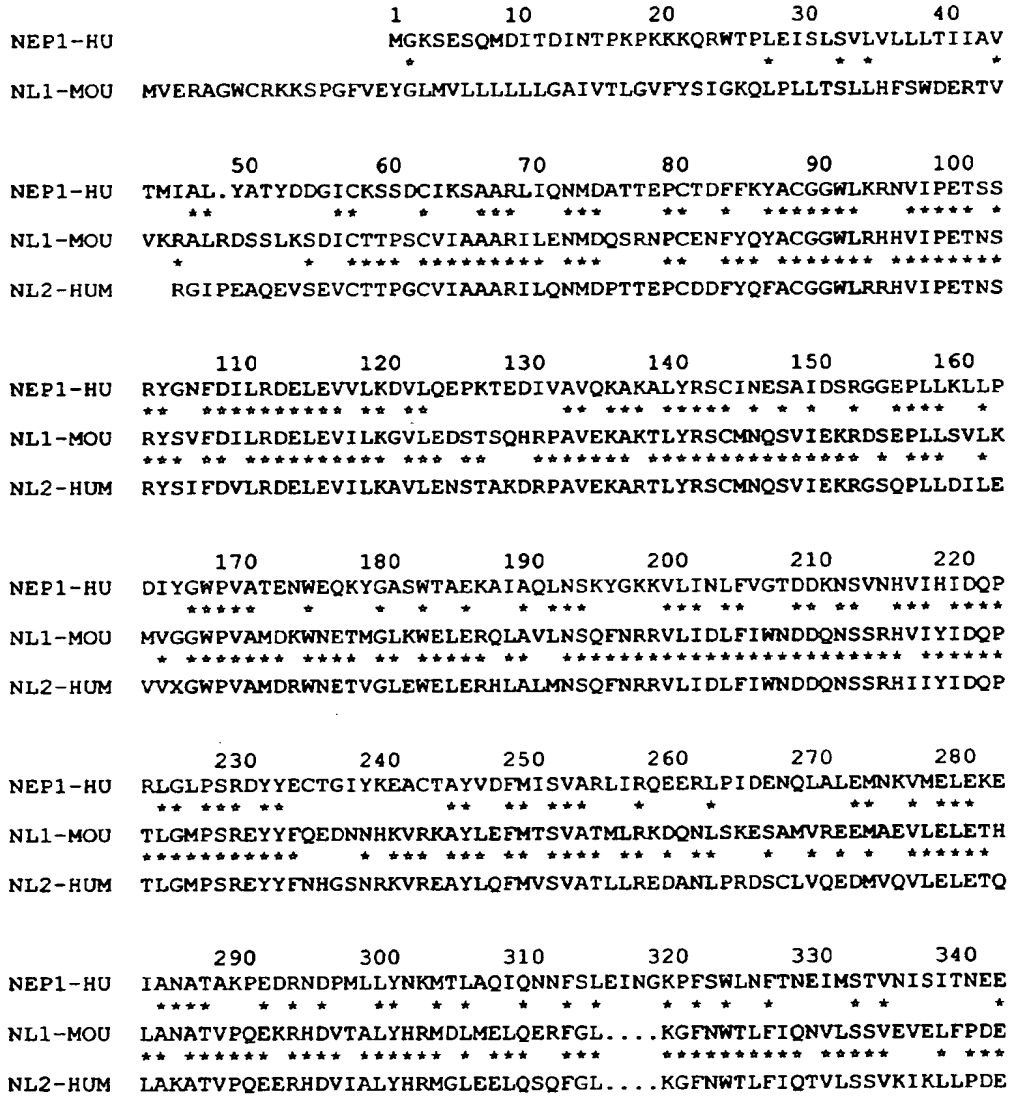
FIGURE 5

Partial sequence of NL-3 from human

```
                                                              20
                              gln arg leu glu glu leu asp trp met asp ala glu thr arg ala ala ala arg ala lys
  1                           CAG CGC CTG GAG GAG CTG GAC TGG ATG GAC GCC GAG ACC AGG GCT GCT GCT CGG GCC AAG
                                                                                                            50
    leu gln tyr met met val met val gly tyr pro asp phe leu leu lys pro asp ala val asp lys glu tyr glu phe glu val his glu
 61 CTC CAG TAC ATG ATG GTG ATG GTC GGC TAC CCG GAC TTC CTG CTG AAA CCC GAT GCT GTG GAC AAG GAG TAT GAG TTT GAG GTC CAT GAG
                                                                                                            80
    lys thr tyr phe lys asn ile leu asn ser ile arg phe ser ile gln leu ser val lys lys ile arg gln glu val asp lys ser thr
151 AAG ACC TAC TTC AAG AAC ATC TTG AAC AGC ATC CGC TTC AGC ATC CAG CTC TCA GTT AAG AAG ATT CGG CAG GAG GTG GAC AAG TCC ACG
                                                                                                           110
    trp leu leu pro pro gln ala leu asn ala tyr tyr leu pro asn lys asn gln met val phe pro ala gly ile leu gln pro thr leu
241 TGG CTG CTC CCC CCA CAG GCG CTC AAT GCC TAC TAT CTA CCC AAC AAG AAC CAG ATG GTG TTC CCC GCG GGC ATC CTG CAG CCC ACC CTG
                                                                                                           140
    tyr asp pro asp phe pro gln ser leu asn tyr gly gly ile gly thr ile ile gly his glu leu thr his gly tyr asp asp trp gly
331 TAC GAC CCT GAC TTC CCA CAG TCT CTC AAC TAC GGG GGC ATC GGC ACC ATC ATT GGA CAT GAG CTG ACC CAC GGC TAC GAC GAC TGG GGG
                                                                                                           170
    gly gln tyr asp arg ser gly asn leu leu his trp trp thr glu ala ser tyr ser arg phe leu arg lys ala glu cys ile val arg
421 GGC CAG TAT GAC CGC TCA GGG AAC CTG CTG CAC TGG TGG ACG GAG GCC TCC TAC AGC CGC TTC CTG CGA AAG GCT GAG TGC ATC GTC CGT
                                                                                                           200
    leu tyr asp asn phe thr val tyr asn gln arg val asn gly lys his thr leu gly glu asn ile ala asp met gly gly leu lys leu
511 CTC TAT GAC AAC TTC ACT GTC TAC AAC CAG CGG GTG AAC GGG AAA CAC ACG CTT GGG GAG AAC ATC GCA GAT ATG GGC GGC CTC AAG CTG
                                                                                                           230
    ala tyr his ala tyr gln lys trp val arg glu his gly pro glu his pro leu pro arg leu lys tyr thr his asp gln leu phe phe
601 GCC TAC CAC GCC TAT CAG AAG TGG GTG CGG GAG CAC GGC CCA GAG CAC CCA CTT CCC CGG CTC AAG TAC ACA CAT GAC CAG CTC TTC TTC
                                                                                                           260
    ile ala phe ala gln asn trp cys ile lys arg arg ser gln ser ile tyr leu gln val leu thr asp lys his ala pro glu his tyr
691 ATT GCC TTT GCC CAG AAC TGG TGC ATC AAG CGG CGG TCG CAG TCC ATC TAC CTG CAG GTG CTG ACT GAC AAG CAT GCC CCT GAG CAC TAC
                                                                                                           290
    arg val leu gly ser val ser gln phe glu glu phe gly arg val leu his cys pro lys val ser pro met asn pro ala his lys cys
781 AGG GTG CTG GGC AGT GTG TCC CAG TTT GAG GAG TTT GGC CGG GTT TTA CAC TGT CCA AAG GTC TCA CCC ATG AAC CCT GCC CAC AAG TGT
            293
    ser val trp
871 TCC GTG TGG
```

FIGURE 6

Sequence comparison between NEP, NL1, NL2 and NL3



```
                350        360        370        380        390        400
NEP1-HU   DVVVYAPEYLTKLKPILTKYSARDLQNLMSWRFIMDLVSSLSRTYKESRNAFRKALYGTT
NL1-MOU   EVVVYGIPYLENLEDIIDSYSARTMQNYLVWRLVLDRIGSLSQRFKEARVDYRKALYGTT
NL2-HUM   EVVVYGIPYLQNLENIIDTYSARTIQNYLVWRLVLDRIGSLSQRFKDTRVNYRKALFGTM

                410        420        430        440        450        460
NEP1-HU   SETATWRRCANYVNGNMENAVGRLYVEAAFAGESKHVVEDLIAQIREVFIQTLDDLTWMD
NL1-MOU   VEEVRWRECVSYVNSNMESAVGSLYIKRAFSKDSKSTVRELIEKIRSVFVDNLDELNWMD
NL2-HUM   VEEVRWRECVGYVNSNMENAVGSLYVREAFPGDSKSMVRELIDKVRTVFVETLDELGWMD
NL3-HUM                                                   QRLEELDWMD

                470        480        490        500        510        520
NEP1-HU   AETKKRAEEKALAIKERIGYPDDIVSNDNK.LNNEYLELNYKEDEYFENIIQNLKFSQSK
NL1-MOU   EESKKKAQEKAMNIREQIGYPDYILEDNNKHLDEEYSSLTFYEDLYFENGLQNLKNNAQR
NL2-HUM   EESKKKVQEKAMSIREQIGHRDYILEETNRRLDEGYSNLNFSEDLYFENSLQNLKVGAQR
NL3-HUM   AETRAAARAKLQYMMVMVGYPDFLLKPDSAVDKEYEFFEVHEKTYFKNILNSIRFSIQL

                530        540        550        560        570        580
NEP1-HU   QLKKLREKVDKDEWISGAAVVNAFYSSGRNQIVFPAGILQPPFFSAQQSNSLNYGGIGMV
NL1-MOU   SLKKLREKVDQNLWITGAAVVNAFYSPNRNQIVFPAGILQPPFFSKDQPQSLNFGGIGMV
NL2-HUM   SLRKLREKVDPNLWIIGAAVVNAFYSPNRNQIVFPAGILQPPFFSKEQPQALNFGGIGMV
NL3-HUM   SVKKIRQEVDKSTWLLPPQALNAYYLPNKNQMVFPAGILQPTLYDPDFPQSLNYGGIGTI

                590        600        610        620        630        640
NEP1-HU   IGHEITHGFDDNGRNFNKDGDLVDWWTQQSASNFKEQSQCMVYQYGNFSWDLAGGQHLNG
NL1-MOU   IGHEITHGFDDNGRNFDKNGNMLDWWSNFSARHFQQQSQCMIYQYGNFSWELADNQNVNG
NL2-HUM   IGHEITHGFDDNGRNFDKNGNMMDWWSNFSTQHFREQSECMIYQYGNYSWDLADGQNVNG
NL3-HUM   IGHELTHGYDDWGGQYDRSGNLLHWWTEASYSRFLRKAECIVRLYDNFT   VYNQRVNG
```

```
                650        660        670        680        690        700
NEP1-HU   INTLGENIADNGGLGQAYRAYQNYIKKNGEEKLLPGLDLNHKQLFFLNFAQVWCGTYRPE
            ***********  *** **         *    **** *   **** * ******* ****
NL1-MOU   FSTLGENIADNGGVRQAYKAYLRWLADGGKDQRLPGLNLTYAQLFFINYAQVWCGSYRPE
          * **********
NL2-HUM   FNTLGENIADNG
            ******** **    ** **   *     *     ** *   *   *****   ** **    *
NL3-HUM   KHTLGENIADMGGLKLAYHAYQKWVREHGPEHPLPRLKYTHDQLFFIAFAQNWCIKRRSQ


                710        720        730        740        750
NEP1-HU   YAVNSIKTDVHSPGNFRIIGTLQNSAEFSEAFHCRKNSYMNPEKKCRVW
           ** *********    *   * ***    *******    * * * * ** *
NL1-MOU   FAVQSIKTDVHSPLKYRVLGSLQNLPGFSEAFHCPRGSPMHPMKRCRIW
             ** * *  ******       *     ***  *** *    *   *
NL3-HUM   SIYLQVLTDKHAPEHYRVLGSVSQFEEFGRVLHCPKVSPMNPAHKCSVW
```

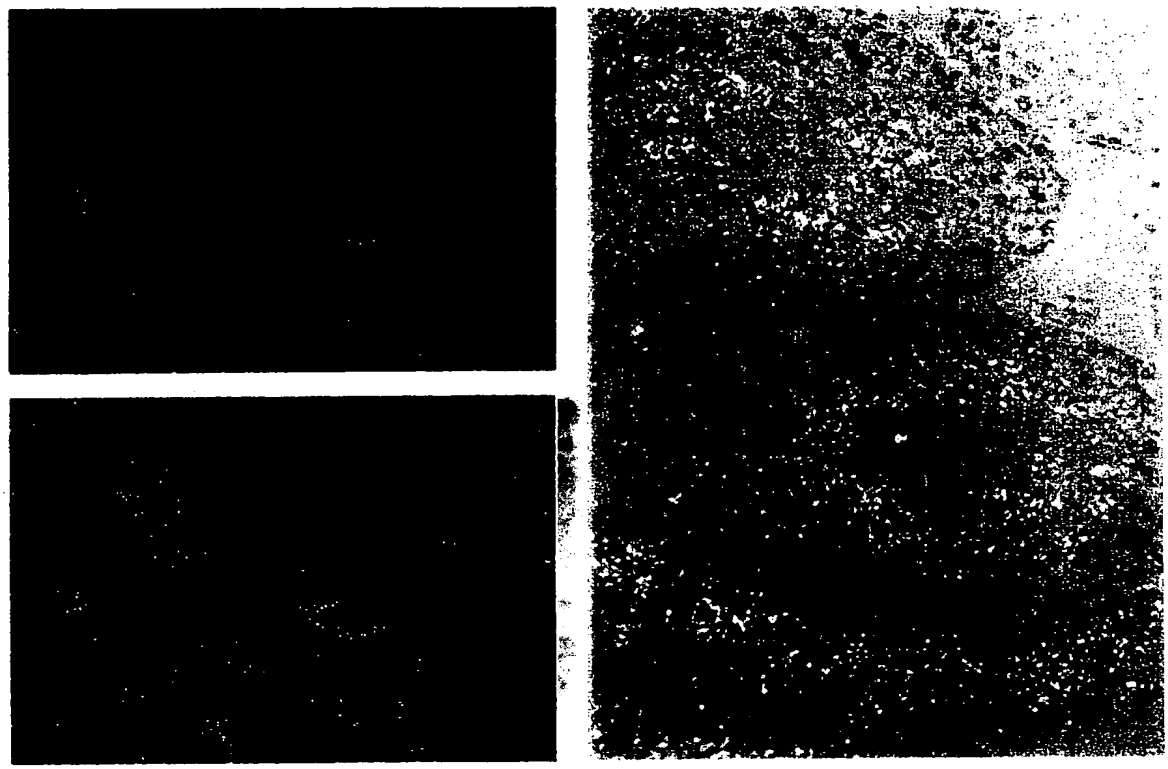


FIGURE 7

Expression of PEX and NL-3 in normal and Hyp mouse embryos

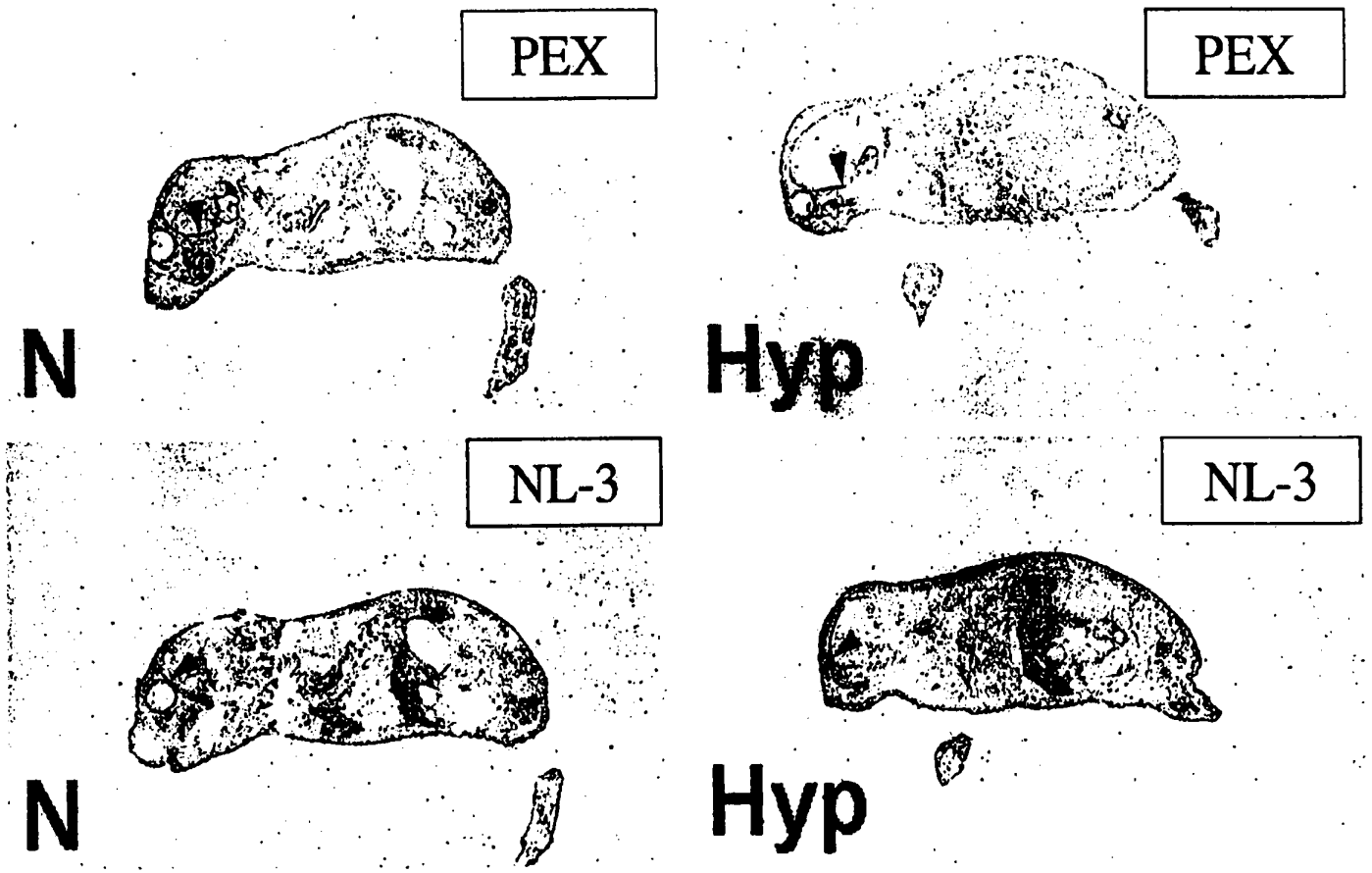


FIGURE 8

NL3 in the BRAIN

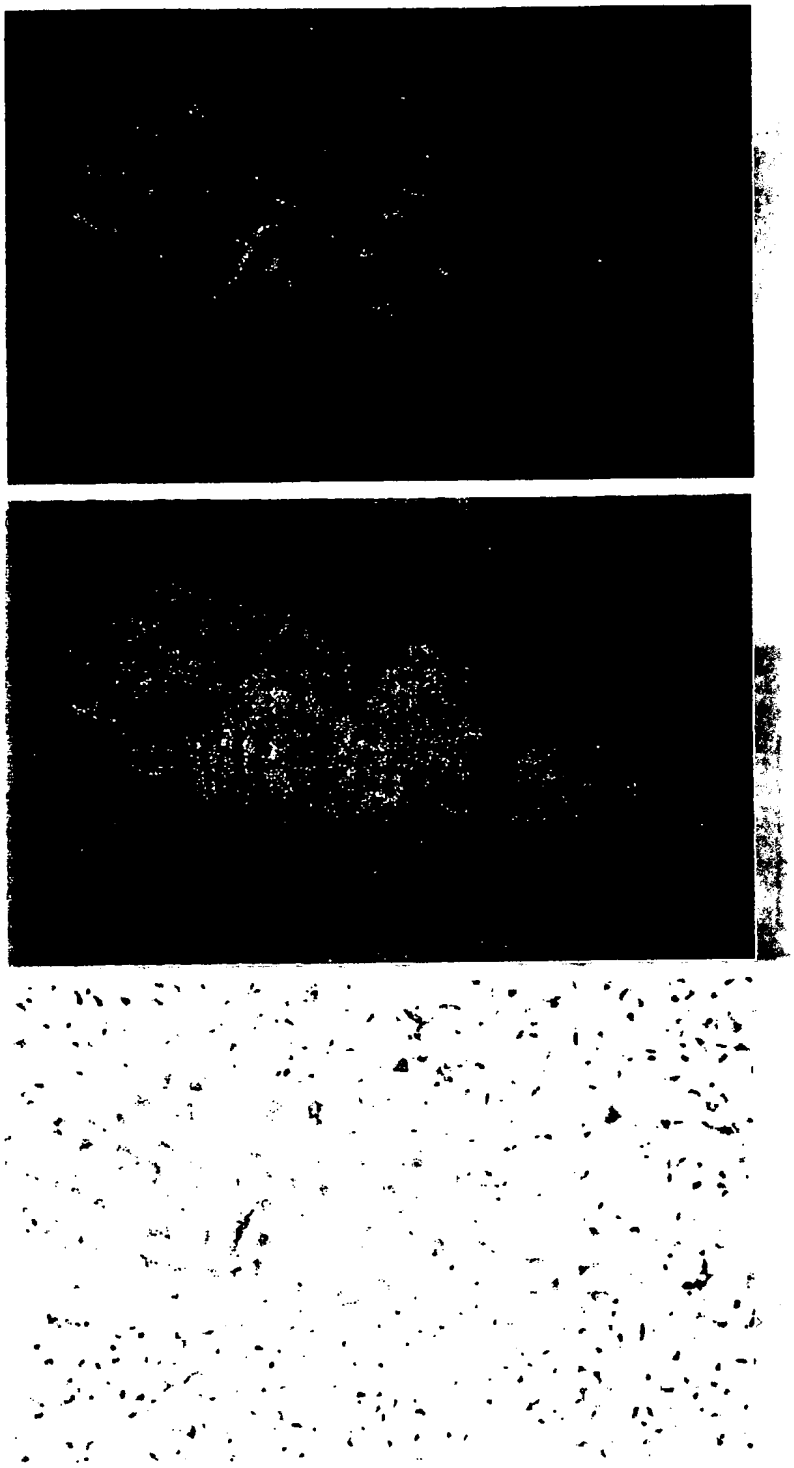
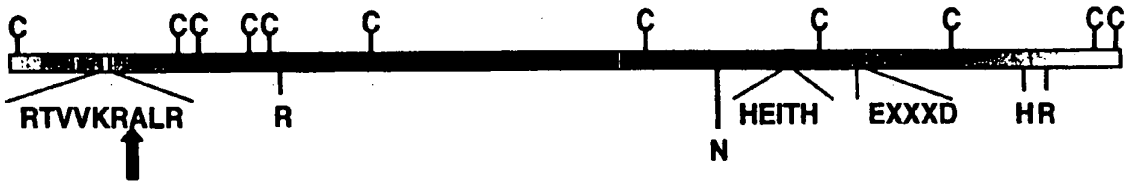
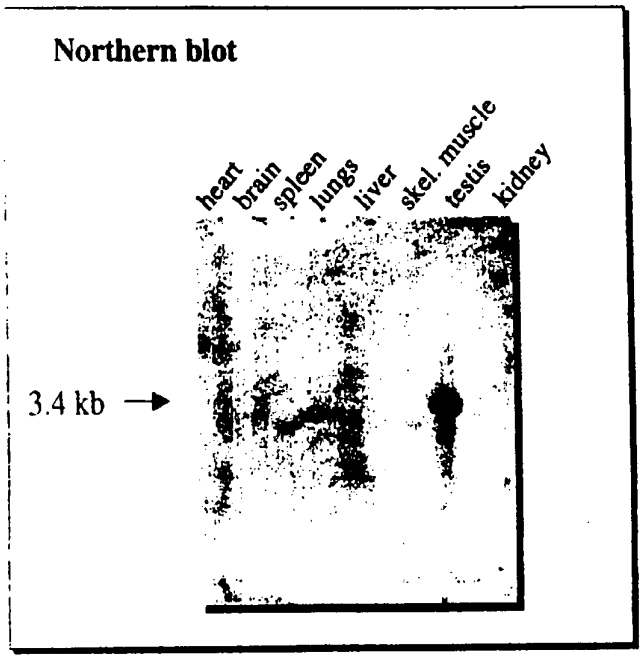


FIGURE 9

FIGURE 10

Structure and expression of NL-1





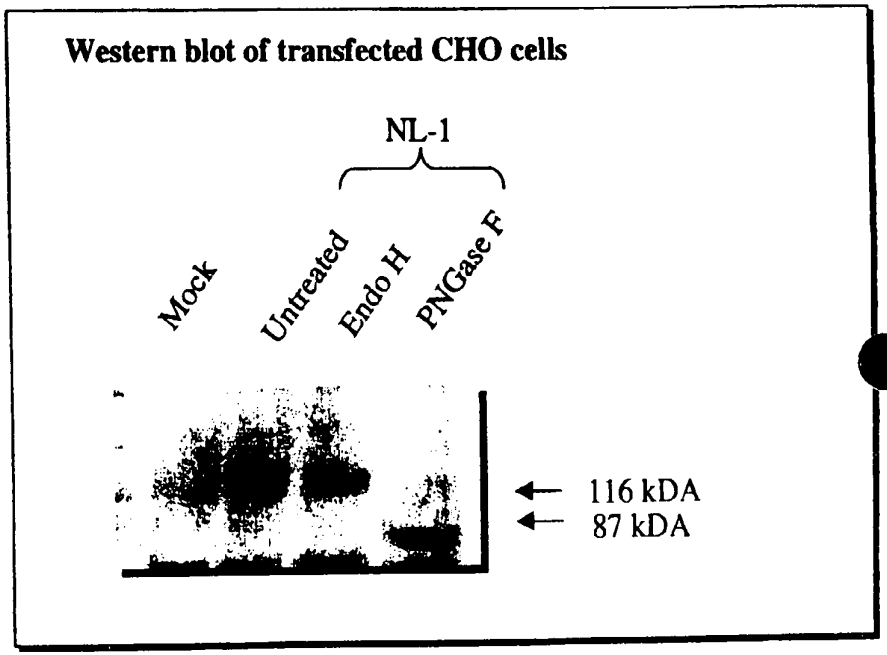
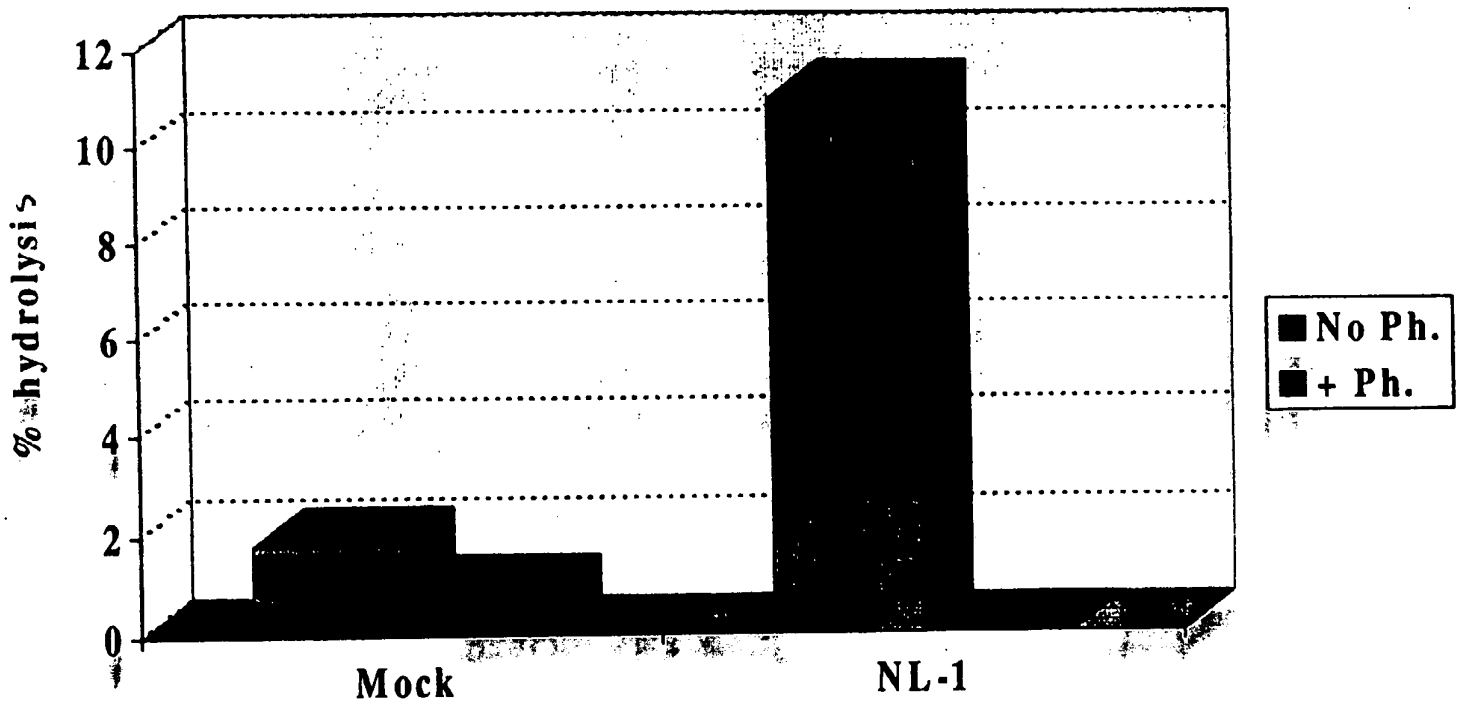


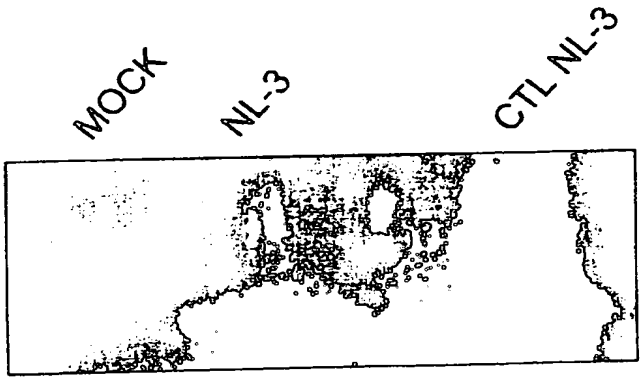
FIGURE 11

Enzymatic activity of NL-1



Degradation of [^{3}H]Tyr, D-Ala$_2$, Leu$_5$-enkephalin

FIGURE 12



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